Chloroplastic stress tolerance in *rcd1* mutant of *Arabidopsis thaliana*

Master's Thesis

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Tiivistelmä — Referat — Abstract

Fluctuating light conditions can cause light stress for plants. The photosynthetic apparatus can be damaged by the excess light. Light stress causes formation of reactive oxygen species in chloroplasts. *Arabidopsis thaliana*'s mutant *radical induced cell death1* (*rcd1*) is tolerant to this stress. In my thesis I used a compound called methyl viologen which causes the formation of reactive oxygen species in chloroplasts. It has been used as a herbicide. By using this compound, we can make the light stress worse and see bigger differences between the *rcd1* mutant and the wild type. We identified the causative gene of *rcd1*'s chloroplastic stress tolerance, clarified the dependence of growth light intensity for chloroplastic stress tolerance and explored possible structural differences at the cellular level between the wild type and *rcd1*. Finding the genes that prevent light stress would allow a light stress tolerant crop production which could make food production easier in hot and dry areas of the world.

My thesis is a part of a screening study where *rcd1* mutants were screened for lowered tolerance to light stress. The amount of stress of the leaves was defined by measuring the chlorophyll fluorescence. Two most promising lines which got damaged by methyl viologen were called #20 and #54. For these a backcrossing was made with the *rcd1*. Clear correlation was found from their offspring between the phenotype and the methyl viologen tolerance. The correlation was strongest in the line #20 so we focused on it. Small and yellowish pale individuals which resembled their parents were the most sensitive to methyl viologen. These individuals were selected for the sequencing. Candidate genes were in the chromosome 3. The most promising one was called AT3G29185 or *BIOGENESIS FACTOR REQUIRED FOR ATP SYNTHASE1* (*BFA1*). We ordered *bfa1* mutant's seeds. We found that *bfa1* mutant was itself sensitive to methyl viologen proving our observation.

We discovered that methyl viologen tolerance is growth light dependent. The individuals that grew under higher intensity of light were more tolerant to methyl viologen in both the wild type and *rcd1* mutant. We didn't find structural differences at the cellular level by confocal microscopy. Thus, they can't explain the differences in the methyl viologen tolerance.

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Arabidopsis (*Arabidopsis thaliana*), *rcd1*, chloroplasts, light stress, reactive oxygen species (ROS), methyl viologen (MV/paraquat), backcrossing, *BFA1*

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Tiivistelmä — Referat — Abstract

Vaihtelevat valo-olosuhteet voivat aiheuttaa kasveille valostressiä. Fotosynteesi voi vaurioitua liian voimakkaasta valosta. Valostressi aiheuttaa happiradikaalien muodostusta viherhiukkasiin eli kloroplasteihin. Lituruohon *radical induced cell death1* (*rcd1*) -mutantti kestää hyvin tätä stressiä. Tutkielmassani käytin parakvatti -yhdistettä, joka niin ikään lisää happiradikaalien muodostusta kloroplasteihin. Tätä yhdistettä on käytetty rikkaruohomyrkyissä. Parakvatilla pystytään pahentamaan valostressiä, ja saamaan mahdolliset erot suuremmiksi *rcd1*:n ja villityypin välillä. Tutkielmassani selvitin *rcd1* -mutantin valostressin sietokyvyn aikaansaavan geenin, kasvuolosuhteiden valon intensiteetin vaikutuksen kloroplastisen stressin sietokykyyn, sekä kartoitin rakenteellisia solutason eroja *rcd1*:n ja villityypin välillä. Valostressiä ehkäisevien geenien löytäminen mahdollistaisi valostressiä sietävien viljelylajikkeiden kehittämisen, mikä taas voisi helpottaa ruoantuotantoa maapallon kuumilla ja kuivilla alueilla.

Tutkielmani on osa skriinaustutkimusta, jossa etsittiin *rcd1* -mutantteja, joilla oli heikentynyt valostressin sietokyky. Stressin määrä saatiin lehdistä selville klorofyllin fluoresenssimittauksella. Kaksi lupaavinta linjaa, joille parakvatti aiheutti eniten vaurioita, olivat nimeltään #20 ja #54. Näille tehtiin takaisinristeytys *rcd1*:n kanssa. Näiden jälkeläisistä havaittiin selvä korrelaatio fenotyypin ja parakvatin sietokyvyn välillä. Linjassa #20 korrelaatio oli selvin, joten keskityimme siihen. Pienet ja kellahtavan väriset yksilöt, jotka muistuttivat fenotyypiltään suuresti #20:n vanhempaansa, olivat myös herkimpiä parakvatille. Nämä yksilöt valittiin sekvensointiin. Kandidaattigeenit sijaitsivat kromosomissa 3. Lupaavin niistä oli nimeltään AT3G29185 eli *BIOGENESIS FACTOR REQUIRED FOR ATP SYNTHASE1* (*BFA1*). Tilasimme *bfa1* -mutantin siemeniä. Havaitsimme, että *bfa1* -mutantti oli itsessään herkkä parakvatille, mikä todisti havaintomme.

Havaitsimme myös, että kasvuolosuhteiden valon intensiteetti vaikuttaa kasvien parakvatin sietokykyyn. Sekä *rcd1* -mutantilla että villityypillä voimakkaammassa valossa kasvaneet yksilöt kestivät parakvattia paremmin. Rakenteellisia eroja ei havaittu *rcd1*:n ja villityypin välillä konfokaalimikroskopialla, joten ne eivät selitä eroa parakvatin sietokyvyssä.

Avainsanat — Nyckelord — Keywords
Lituruoho (*Arabidopsis thaliana*), *rcd1*, kloroplastit, valostressi, happiradikaalit, parakvatti, takaisinristeytys, *BFA1*

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CONTENTS

ABBREVIATIONS & EXPLANATIONS

MV = Methyl viologen = Paraquat. An organic compound with redox activity. Creates reactive oxygen species in the chloroplasts by accepting electrons from Photosystem I and transferring them to molecular oxygen.

Col-0/Columbia = One of the wild type ecotypes of *Arabidopsis thaliana* that is used in my study.

rcd1 = radical-induced cell death1 -mutant of *Arabidopsis thaliana*.

PAM = Pulse-amplitude modulation chlorophyll fluorometer. Machine to measure variable chlorophyll fluorescence.

Qa = One of the components of the electron transfer chain in chloroplasts.

ROS = Reactive oxygen species.

PS = Photosystem. Protein-pigment complexes involved in photosynthesis.

M2 = Second generation of plants after mutagenesis.

Backcrossing = Crossing of a hybrid with one of its parents.

BcF2 = Backcross F2. Second generation of plants after backcrossing.

NPQ = Non-photochemical quenching. Dissipation of excess excitation energy as heat through molecular vibrations.

µE = Microeinstein. 1 Einstein = 1 mole of photons. We usually measure photosynthetically active radiation in micromoles of photons falling per 1 second on 1 square meter of the surface (µE m^{-2} s^{-1}).

RATIONALE OF THE STUDY

Vascular plants have complicated relationships between photosynthesis and respiration. Photosynthesis occurs in the chloroplasts, while respiration occurs in the mitochondria. Many genes encoding the components of both chloroplasts and mitochondria are localized in the nucleus. In the vascular plant Arabidopsis, RCD1 protein is localized in the nucleus. It binds to and inhibits many different transcription factors. Among them are the transcription factors that mediate mitochondrial signalling (ANAC013 and ANAC017). So, when RCD1 is missing (in the *rcd1* mutant), that mitochondrial signalling is always active. Thus, *rcd1* mutant has defects in mitochondria. But also, surprisingly, *rcd1* mutant has been shown to have defects in the chloroplasts. For example, it is tolerant to chloroplast stress (Fujibe et al., 2004 & 2006; Shapiguzov et al., 2019). In particular, *rcd1* mutant is very tolerant to the compound called methyl viologen (MV, see "Abbreviations and explanations"). How the defects in mitochondria of *rcd1* affect the chloroplasts remains mysterious. We are trying to identify the causative genes that could be part of this regulation. By using MV we can increase production of reactive oxygen species (ROS, see "Abbreviations and explanations") in the chloroplasts. In these conditions the physiological difference between the wild type and *rcd1* plants becomes very clear. We study performance of *rcd1* by measuring photosynthesis (section 1.2). We do this by *in vivo* imaging of chlorophyll fluorescence (section 1.3). For our studies we are using the model plant Arabidopsis, ecotype Col-0 (section 1.1). In general, these studies help us to understand how respiration and photosynthesis affect each other within the plant cell. Additionally, finding the genes involved in MV tolerance could be beneficial also for crop production. Chloroplastic ROS are also generated without any MV, during normal growth conditions, or light stress. So, *rcd1* mutant and MV treatment are the experimental tools to study how plants adjust to light stress. A light stress tolerant crop may improve yields in hot and dry areas of the world and thus prevent famines.

1 INTRODUCTION

1.1 *Arabidopsis thaliana*

The thale cress or Arabidopsis (*Arabidopsis thaliana*) is an annual plant belonging to *Brassicaceae* family. Arabidopsis is a flowering weed plant native to Eurasia and Africa. It has been widely used in cell biology research (Meinke et al., 1998) and thus can be described as a model organism. Arabidopsis was the first plant which genome was sequenced. The project was finished in 2000 (The Arabidopsis Genome Initiative, 2000). The genome size is 125 megabases. It contains five chromosomes including about 25000 genes encoding proteins. Advantages for studies are its small size, short life cycle, easy growing, massive seed production and small genome. In addition, Arabidopsis provides numerous research tools including a huge collection of available mutants. Not only plant biology benefits from the research made in Arabidopsis but also agriculture, evolutionary biology, bioinformatics, chemistry, genomics and medicine. The most commonly used wild type ecotype of Arabidopsis is Col-0.

1.2 Light absorption and photosynthesis

Photosynthesis allows plants to convert light energy into biochemical energy in chloroplasts. For photosynthesis, plants mostly use visible part of the light with the wavelength 400-700 nm. Chlorophyll molecules are the pigments that absorb light that is used for photosynthesis. These molecules are part of antenna complexes (or light-harvesting complexes) together with proteins in the thylakoid membrane of a chloroplast. Antenna complexes capture light energy and transfer it to a reaction center of a photosystem. Reaction centers convert excitation energy to chemical energy. They are also formed from pigments and proteins. Most photosynthesizing organisms have two types of photosystems. A captured photon ultimately excites an electron in photosystem II (PSII). The excited electrons are then transferred through a series of redox reactions to photosystem I (PSI). This gives rise to the chloroplast electron transfer chain. Electron transfer fuels accumulation of protons inside thylakoids. The energy of

these protons is used to synthesize ATP. In PSII, the oxygen evolving complex (OEC) splits a water molecule into oxygen, protons and electrons that refill the supply of electrons (Antal et al., 2013). The electrons of the electron transfer chain are finally used to reduce various targets, including reduction of NADP+ molecules to NADPH. Both ATP and reduced NADPH are needed for carbon fixation in Calvin cycle (Liu et al., 2004; Porcar-Castell et al., 2014). Another chloroplast electron acceptor is molecular oxygen. It is reduced by excited electrons forming chloroplastic ROS.

1.3 Studying photosynthesis with the help of chlorophyll fluorescence

Chlorophyll fluorescence is a by-product of photosynthesis. It can be used as an indicator of photosynthetic energy conversion and stress. It is used to study photosynthetic performance (Baker, 2008). After the pigments of PSII have captured the light, there are three ways for the light energy to be relaxed. The energy can be used to drive the photosynthesis and the electron transfer chain (photochemical quenching) or it can be released as heat (non-photochemical quenching; NPQ) or chlorophyll fluorescence (Porcar-Castell et al., 2014; Baker, 2008). Fluorescence is radiative loss of the energy of absorbed photons. The sum of these three processes is always equal to the total light excitation energy. By relying on this fact, we can estimate the amount of these processes by measuring the fluorescence of the leaves *in vivo*. Fluorescence gives information on the state of PSII, electron transfer, NPQ and other processes in the chloroplast. PSII is sensitive to stress. Its damage is frequently the sign of the leaf's stress. Damage to PSII is most frequently estimated by measuring the chlorophyll fluorescence parameter Fv/Fm. It is also called the maximal photochemical yield of PSII (Baker, 2008). Fv/Fm comes from (Fm-F0)/Fm. Fm is the maximal fluorescence level. It is the level of fluorescence when the PSII electron acceptor Qa is maximally reduced, so it cannot accept more electrons. Thus, the electron transfer chain is blocked. Fm is measured with the help of a saturating light flash – a flash of short, usually 1 second, and very intensive light. F0 is the minimal fluorescence level. It is measured in darkness, when Qa is maximally oxidized. F0 is measured with very weak measuring light flash. The difference between Fm and F0 is defined as the variable fluorescence, Fv. Fv/Fm is used to estimate

the maximum quantum yield of Qa reduction and thus PSII photochemistry, i.e., what proportion of the captured photons gave rise to excited electrons. Under many stress conditions, Fv/Fm is affected (Baker, 2008). In light NPQ turns on and plant dissipates excess excitation energy as heat (Porcar-Castell et al., 2014). Thus, the yield of fluorescence gets lower. For this reason, to accurately measure Fv/Fm one needs to adapt plants to darkness. In the darkness NPQ gradually turns off. Therefore, a dark adaptation (at least 20 minutes) is needed to dissipate NPQ and measure maximal yield of PSII (Baker, 2008).

We perform the imaging of chlorophyll fluorescence using the pulse amplitude modulation (PAM) fluorometry (see section "MATERIALS AND METHODS").

In addition to NPQ, there exist many other regulations of photosynthesis that occur at the levels higher than the chloroplast. For example, expression of chloroplast-localized proteins can be changed in the nucleus. Plants exchange gases and evaporate water through stomata, regulating gas supply to chloroplasts and leaf temperature. Plants can move leaves and adjust their angle to regulate the absorbed light energy. These regulations help plant to adapt both to insufficient and to excessive light. During the so-called shade avoidance response, the plants grow towards the source of light. Chloroplasts can move to a favorable position to avoid excessive light. Plants can produce more pigments for example anthocyanins to protect photosynthesis by screening the light.

1.4 Reactive oxygen species

Reactive oxygen species (ROS) are reactive molecules that contain oxygen. This group includes superoxide (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical ($^{\bullet}OH$), and singlet oxygen ($^1O_2$) (Noctor & Foyer, 2016). They are formed when energy or electrons are transferred to oxygen. They are byproducts of the metabolism that involves oxygen. ROS are widely produced in different cellular compartments such as plasma membrane, cell wall, mitochondria, chloroplasts, peroxisomes and glyoxysomes. ROS are important signalling molecules for the adaptation and acclimation of plants to their environment (Shapiguzov et. al., 2012; Waszczak et al., 2018).

Chloroplastic ROS are produced in photosynthesis and are associated with light (Waszczak et al., 2018). In light, photosynthesis is the major source of ROS in plant cells (Fryer et al., 2003). Interestingly, ROS function in plant signalling and defence responses. For example, chloroplast generated ROS can up-regulate defence-related genes, down-regulate photosynthesis genes and limit the spread of cell death (Straus et al., 2010). However, different types of ROS are produced in different locations of the chloroplast electron transfer chain. For example, singlet oxygen is produced by PSII and promotes programmed cell death, while superoxide is produced by PSI and may in some cases counteract cell death (Shapiguzov et al., 2012).

The main source of ROS in the chloroplast is PSI (Noctor & Foyer, 2016). Part of the electrons is transferred from PSI to oxygen at the electron-acceptor side of PSI, which causes the formation of superoxide. This superoxide is quickly converted to hydrogen peroxide. In photosynthesis the reduction of O_2 by PSI and photorespiration cause increased production of H_2O_2 (Mullineaux et al., 2006). Increased production of ROS in the chloroplast ultimately inhibits the repair of PSII leading to decreased maximal photochemical yield (Fv/Fm) of PSII. Fluctuating light also causes overproduction of ROS in chloroplasts (Allahverdiyeva et al., 2015). In this case, the potential target of damage is PSI. Enzymatic and non-enzymatic scavenging systems control the level of ROS in the chloroplast (Allahverdiyeva et al., 2015; Pospisil, 2016).

Methyl viologen/MV/paraquat is an organic compound that has redox activity. It is used in herbicides because it produces superoxide anions in the chloroplast under light. It is catalyzing the above-described transfer of electrons from PSI to molecular oxygen. We still don't know exactly how MV kills a plant. This ROS is also formed without MV, but much slower. We used MV to make the chloroplast ROS formation worse. We use MV-tolerant mutant *rcd1* to study the mechanisms of MV toxicity and tolerance.

1.5 *rcd1* mutant

RCD1 gene (At1g32230) of *Arabidopsis thaliana* was first described in 2000 (Belles-Boix et al., 2000; Overmyer et al., 2000). RCD1 is an important plant-specific regulator of stress and hormonal and developmental responses in *Arabidopsis* (Jaspers et al., 2009;

Shapiguzov et al., 2019). It is exclusively restricted to plants (Belles-Boix et al., 2000). RCD1's protein family is called SIMILAR TO RCD-ONE (SRO) (Jaspers et al., 2009). In my study I used *rcd1-4* mutant that completely lacks *RCD1* expression. It is a T-DNA allele of *rcd1*. There also exist other loss-of-function alleles or RCD1, however all the corresponding *rcd1* mutants have very similar phenotypes.

Mutants are widely used in plant physiology research. *rcd1* has several developmental defects. For example, *rcd1* rosette is smaller than the wild type and has curlier leaves. This mutant has been isolated in a lot of stress-related genetic screens. Importantly for this study, *rcd1* mutant overexpresses mitochondrial dysfunction stimulon (MDS) genes. Normally RCD1 protein inhibits MDS signalling pathway. The mutant has also alterations in the chloroplasts (Shapiguzov et al., 2020).

The mitochondrial defects of this mutant have already been mentioned above ("Rationale of the study"). Most importantly for my study, *rcd1* is very tolerant to MV (Fujibe et al., 2004 & 2006; Shapiguzov et al., 2019). Light stress causes ROS formation in chloroplasts. *rcd1* is also more tolerant to UV-B irradiation than the wild type (Fujibe et al., 2004) and to high light (Cui et al., 2019). In other words, *rcd1* is more tolerant to light stress. We are trying to understand what genes could be involved in the light stress tolerance and MV tolerance of the *rcd1* mutant.

1.6 Chloroplast ATP synthase and BFA1

Our study has revealed *BIOGENESIS FACTOR REQUIRED FOR ATP SYNTHASE1* (*BFA1*) (Zhang et al., 2018) as one of the possible genes that make *rcd1* tolerant to MV.

BFA1 protein is localized to the chloroplast stroma where it assists the assembly of the chloroplast ATP synthase (Zhang et al., 2018). F-type ATP synthases provide most of the cellular ATP using transmembrane proton gradient formed by electron transport chains. The synthase contains two rotary motors, F_1 and F_0. In the *bfa1* mutant assembly of the F_1 module of ATP synthase is impaired. Thus, *bfa1* has reduced activity of the chloroplast ATP synthase.

2 MATERIALS AND METHODS

2.1 Growth conditions

Plants were grown under fluorescent lamps at 12-hour photoperiod for the first three weeks of growing. The light intensities on the cultivation shelves were 220 or 280 µE depending on the purpose. PAM measurements were done after three weeks. If the plants were needed for seed production, they were then placed to a greenhouse compartment under 16-hour light photoperiod, where high pressure sodium lamps were used. There the light intensity for the plants was at least 150 µE. Plants were cultivated on soil, which contained peat and vermiculite 1:1.

2.2 The principle of forward genetic screens

The idea of all genetic suppressor screens is that we have line X that has some interesting properties. To find the reasons, we make random mutagenesis of this line X. We made this mutagenesis in the *rcd1* mutant using a chemical ethyl-methanesulfonate (EMS), which resulted in hundreds of spontaneous point mutations scattered across the genome. Then we propagate the plants to M2. In this generation some of the produced mutations may become homozygous according to Mendel's laws, thus revealing the phenotype of recessive mutations. In M2 we search for the individuals, in which the interesting properties of the line X are broken. After this, we take these individuals and look for what gene was broken. Then we do the sequencing and discover the causative gene (James et al., 2013). Thus, we can find the gene that is contributing to the interesting properties of our line X. In our case, we wanted to find out what caused MV tolerance of *rcd1*.

2.3 Backcrossing and mapping

Backcrossing means crossing of a hybrid with one of its parents. The goal is to achieve offspring with a genetic identity which is closer to that of the parent. Each generation of

backcrossing increases the percentage of parental alleles (Allen et al., 2013). If the DNA sequencing was done directly from the M2 generation, we wouldn't know which of the hundreds of existing EMS mutations caused the phenotype we are interested in. When the M2 generation is crossed with the parental non-mutagenized line X, all mutations will segregate in F2 independently according to Mendel's laws. From BcF2 we will select only those that again show the phenotype that we want. From the pool of those individuals a nuclear DNA is extracted and sent to sequencing. Since DNA was pooled from different BcF2 plants, some of these reads will be coming from the mutagenized grandparent, and some from the non-mutagenized (= line X) grandparent. But since we selected only those BcF2 plants that again showed the phenotype that we want, we will in theory have 100 % of mutagenized reads over the EMS point mutation we are looking for, and only 50 % over all other EMS point mutations.

2.4 Nuclear DNA extraction

The principle of the nuclear DNA extraction is essentially described in Schneeberger et al., 2009. We used CTAB method for the nuclear DNA extraction. CTAB based extraction buffers are common for purifying DNA from plants. This buffer effectively eliminates polysaccharides and polyphenols, which are problematic contaminants.

2.5 Sequencing

The nuclear DNA was sent to FuGU (http://www.helsinki.fi/fugu/) for sequencing.

2.6 Analyses of chlorophyll fluorescence using PAM

Chlorophyll fluorescence measurements were done with Imaging PAM Chlorophyll Fluorometer. Its camera is picking the signal in the far-red area (~650-750 nm) where chlorophyll is fluorescing. For every image it takes two photos: first a control photo, and then a photo under a "measuring pulse" - a flash of weak blue light to excite chlorophyll

fluorescence. Then the software subtracts the first photo from the second, which gives an image of only fluorescence that came from chlorophyll.

We used PAM for taking fluorescence photos of leaf discs floating in 96-well plates. The wells were filled with Milli-Q water with added Tween 20 (0.05%) with or without MV (0,05-2 µM). Tween 20 is a detergent that makes MV solution soapier and thus allows it to leak better inside the leaf. Leaf discs were cut from the leaves and placed into the wells of a plate. The discs were incubated in the darkness overnight in order to allow good penetration of MV into the leaf tissue. Importantly, in darkness MV didn't work, because there was no electron transfer through PSI. In the morning, the plate was moved to PAM and subjected to the cycling light protocol as described in (Shapiguzov et al., 2019). In short, 1-hour light cycles were stimulated to drive photosynthesis and thus chloroplast ROS formation. After each 1 hour of light, 20 minutes of darkness were introduced to relax NPQ. Then maximal Fm was measured to determine quantum yield of PSII (Fv/Fm), and then the next 1 hour of light was started.

2.7 Confocal microscopy

We tested the hypothesis that in *rcd1* the tolerance to MV was due to altered organelle positioning. For that, we used confocal microscopy. Chloroplasts can be seen in the confocal microscope without any staining due to chlorophyll fluorescence. To be able to visualize nuclei *in vivo*, we crossed *rcd1* with the line that expressed YFP (Yellow Fluorescent Protein) -tagged histones (Campilho et al., 2006). YFP's excitation peak is 514 nm and its emission peak is 527 nm (Nagai et al., 2002).

To be able to see mitochondria, we used MitoTracker. MitoTracker is a commercially available fluorescent dye (Chazotte, 2011). It is used for labeling active mitochondria. The method utilizes the mitochondrial membrane potential. MitoTracker enters to the mitochondria only when electrochemical gradient exists on the mitochondrial membrane.

2.8 Western blotting

Western blotting was performed essentially as described in Shapiguzov et al., 2019. The anti-BFA1 antibodies were kindly provided by Prof. Lianwei Peng and Prof. Jean-David Rochaix (Zhang et al., 2018).

3 RESULTS AND DISCUSSION

3.1 Organelle positioning

Could the MV tolerance of *rcd1* be explained by the defect in organelle positioning? To answer this question, we examined the differences between organelle shapes and positioning in Col-0 and in *rcd1*. To be able to visualize nuclei *in vivo*, we crossed *rcd1* with a line expressing YFP (Yellow fluorescent protein)-tagged histones (Campilho et al., 2006).

The progeny with *rcd1* background we have identified by curly leaves. Selection of homozygous *rcd1* : Histone-YFP progeny of the cross is presented in **Table 1**.

Table 1. Segregation of YFP signal in F3 of *rcd1* cross to the YFP-tagged histone line. Positive (Pos.) individuals had their nuclei shining yellow while negatives (Neg.) did not. If all plants of a line were positive, the line was considered as positive homozygous. Otherwise the line was considered as heterozygous or negative homozygous. For example, the line *rcd1* : Histone-YFP #01 is positive because in all its tested progeny nuclei are shining yellow. The line *rcd1* : Histone-YFP #02 is negative, as their nuclei don't shine yellow. Progeny of several F2 individuals of all lines was analysed. 7 of 16 lines were negative. 3 of 16 lines were positive. 6 of 16 were segregating: some seedlings of the line were negative, some of them were positive.

F2 plant #	YFP expression in F3 progeny					Conclusion
	#1	#2	#3	#4	#5	
1	**Pos.**	**Pos.**	**Pos.**	**Pos.**	**Pos.**	**Homozygous**
2	Neg.	Neg.	Neg.	Neg.	Neg.	Homozygous
3	Neg.	Neg.	Neg.	Neg.	Neg.	Homozygous
4	Neg.	Pos.	Pos.	Pos.	Neg.	Heterozygous
5	Pos.	Neg.	Neg.	Pos.	Pos.	Heterozygous
6	Neg.	Neg.	Neg.	Neg.	Neg.	Homozygous
7	Neg.	Pos.	Pos.	Pos.	Pos.	Heterozygous
8	Neg.	Neg.	Neg.	Neg.	Neg.	Homozygous
9	Neg.	Neg.	Neg.	Neg.	Neg.	Homozygous
10	**Pos.**	**Pos.**	**Pos.**	**Pos.**	**Pos.**	**Homozygous**
11	Pos.	Pos.	Pos.	Pos.	Neg.	Heterozygous
12	Neg.	Neg.	Neg.	Neg.	Neg.	Homozygous
13	**Pos.**	**Pos.**	**Pos.**	**Pos.**	**Pos.**	**Homozygous**
14	Pos.	Neg.	Neg.	Pos.	Pos.	Heterozygous
15	Pos.	Pos.	Pos.	Pos.	Neg.	Heterozygous
16	Neg.	Neg.	Neg.	Neg.	Neg.	Homozygous

We compared the obtained *rcd1* : Histone-YFP lines with Col-0 : Histone-YFP plants under the confocal microscope (**Figure 1**). Chloroplasts were observed by the chlorophyll autofluorescence. Nuclei were seen because of YFP fluorescence of the nuclear proteins histones tagged with YFP. In addition, we applied MitoTracker to highlight mitochondria. We were not able to detect any major differences between the wild type and the *rcd1* mutant in terms of shapes and positions of the organelles. We then checked whether treatment with MV had effect on shapes or positions of the organelles. After MV treatment, MitoTracker stopped entering mitochondria both in Col-0 and *rcd1*, but no significant difference between the genotypes was observed.

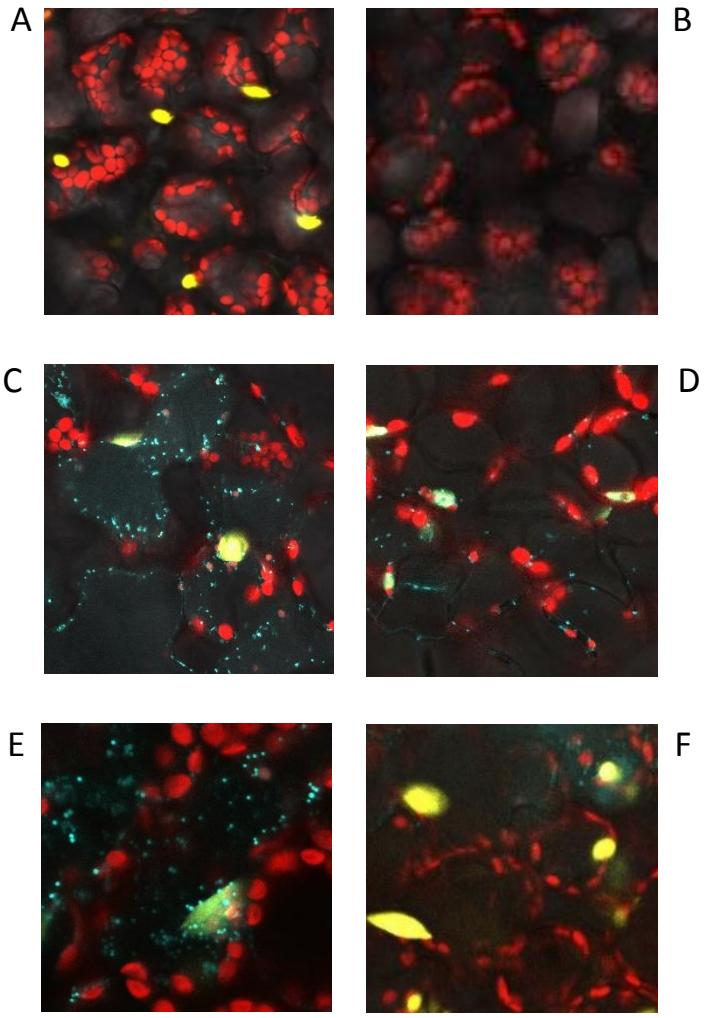

Figure 1. Analysis of organelle positioning in the lines expressing YFP-tagged histones in the *rcd1* background. (A) YFP-positive individual. Nuclei are shown yellow (Histone-YFP), chloroplasts in red (chlorophyll autofluorescence). (B) YFP-negative individual. Organelle positioning was assayed in presence of MitoTracker to reveal mitochondria (blue): (C) Col-0, no MV. (D) Col-0, with MV. MitoTracker staining of mitochondria has largely disappeared. (E) *rcd1*, no MV. (F) *rcd1*, with MV. No reproducible difference between the genotypes was detected either in organelle positions, or in the effect of MV on MitoTracker staining.

3.2 MV *rcd1* suppressor screen

As an alternative approach, we performed a large forward genetic screen to find the genes involved in MV tolerance of *rcd1*. My thesis is part of this big *rcd1* suppressor screen study. In this screen, mutagenized progeny of *rcd1* was screened for increased sensitivity to MV. The lines with restored sensitivity to MV were retained. Then they were crossed to the background *rcd1* to find the mutation.

Mutations were caused to *rcd1* seeds with EMS. The second generation of progeny after the mutagenesis (M2) was used for the MV screen. The *rcd1* mutant is known for its tolerance to high light (Cui et al., 2019). We hypothesized that high light tolerance of *rcd1* is related to its MV tolerance. So, to focus on chloroplast-related functions, we performed the suppressor screen in two steps. Firstly, in total 14 360 M2 seedlings were examined in a high light treatment. From those, candidate plants were retained, that showed decreased tolerance to high light. Next, in the selected individuals MV tolerance was estimated. To test MV stress tolerance, leaf discs were cut and placed onto MV solution. PAM protocol was used to trigger and measure MV toxicity, as described in Shapiguzov et al., 2019. In total, 129 lines were retained which had lowered high light and MV stress tolerance. Those were let to grow to produce self-pollinated seeds. Plants from these seeds (M3 generation) were again taken to MV treatment to confirm the phenotype.

For my research, lines #20 and #54 have been selected for analyses, as in these lines MV tolerance of *rcd1* was largely suppressed. To find the causative mutation among hundreds of EMS-induced point mutations, backcrossing to the parental *rcd1* line was performed.

3.3 MV-sensitive screen candidates

rcd1 is tolerant to ROS produced in chloroplast and thus tolerant to MV. In the genetic screen, we were looking for lines in which MV tolerance of *rcd1* has been suppressed. Thus, these lines became more sensitive to MV than *rcd1*. Our aim was to find the causative gene in these lines. Lines #20 and #54 have partially lost their tolerance to MV.

These are our candidate lines (**Figure 2**). In addition to lower MV tolerance both lines had altered habitus: smaller size and paler leaves (**Figure 3**).

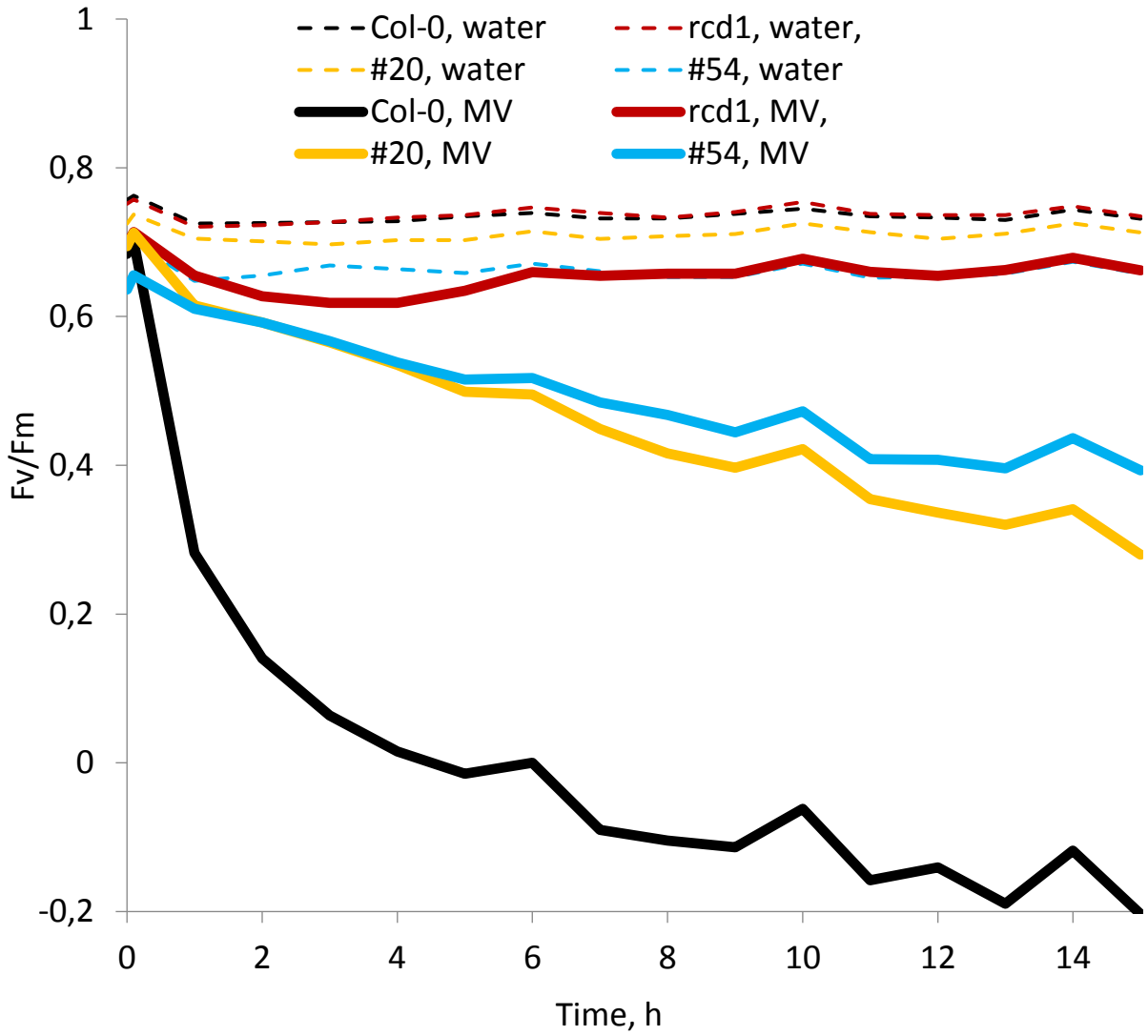

Figure 2. Changes in PSII quantum yield (y-axis) in response to light (hours of light in the x-axis) with 1 µM MV (MV) or without it (water). Col-0 is sensitive to MV, so Fv/Fm drops with time. The *rcd1* mutant is tolerant to MV, thus Fv/Fm remains virtually unchanged. Lines #20 & #54 have mutations that cause a loss of MV tolerance of the *rcd1* background. This is the reason these lines were selected for this study.

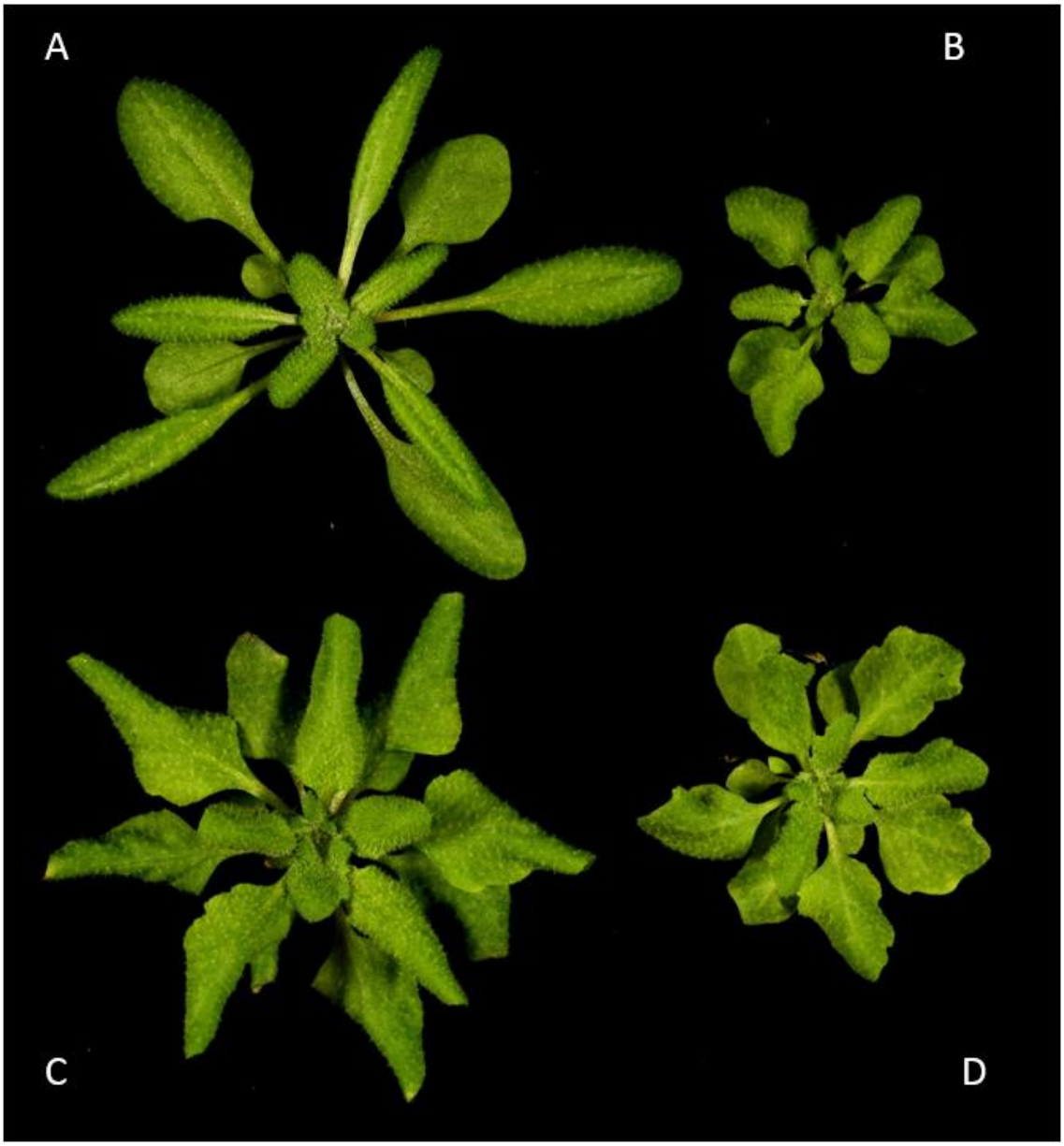

Figure 3. The habitus of the studied plant lines: (A) Col-0. (B) #20. (C) *rcd1*. (D) #54.

3.4 Growth light affects MV sensitivity

There was unknown variation in the MV sensitivity of line #20 (**Figure 4**). We hypothesized that MV sensitivity could depend on the intensity of growth light. To examine this, we made a light intensity test. Plants were grown in different positions of the cultivation shelf. Therefore, the plants received different amount of light (**Figure 5A**). The actual light intensity was measured from every point where the plants were growing. The light intensity was highest in the middle of the shelf and lowest towards the sides. Then we cut leaf discs from these plants and performed PAM-based measurement of MV tolerance as shown in **Figure 2**.

As expected, the results showed us the dependence of MV sensitivity on growth light in the line #20. The leaves were more resistant to MV when they were grown under stronger light (**Figure 5B**).

Interestingly, similar effect was also observed in the wild type Col-0. Col-0's MV sensitivity was growth light dependent as well. Note that since the MV tolerance of Col-0 is lower than that of #20, lower MV concentration was used for this assay (**Figure 6**). Both Col-0 and #20 were more tolerant to MV when they grew under higher light intensities. Probably plants adapt to increased light by producing pigments such as anthocyanins, which protect the photosynthesis. Therefore, for our screening of BcF2 we made sure that the plants were grown under light of the same intensity.

3.5 Backcrossing – Co-segregation of phenotypes and genome resequencing

We next performed backcrossing of line #20 with *rcd1-4* and of line #54 with *rcd1-4*. The aim and the design of the backcrossing is described in "MATERIALS AND METHODS". From BcF2 we needed to select the individuals with decreased MV tolerance. Both #20 and #54 lines were characterized by smaller and paler (yellowish) plant rosettes. We were not sure whether this phenotype was due to the same EMS mutation that also caused MV tolerance. To test if this was the case, we performed the linkage test. We chose 48 dwarf and pale individuals from BcF2 generation and tested the tolerance of these plants to MV. We saw that the visual phenotype correlated with MV tolerance in

all 48 plants for each line. Thus, it was likely that MV tolerance and the visual phenotype were caused by the same mutation. This simplified our selection of BcF2 positive plants, because instead of selecting for MV sensitivity we from then on selected for small and pale phenotype (**Figure 7**). Those plants were pooled together for the nuclear DNA extraction (described in "MATERIALS AND METHODS"). The isolated nuclear DNA was sent for genome resequencing to define the causative mutations.

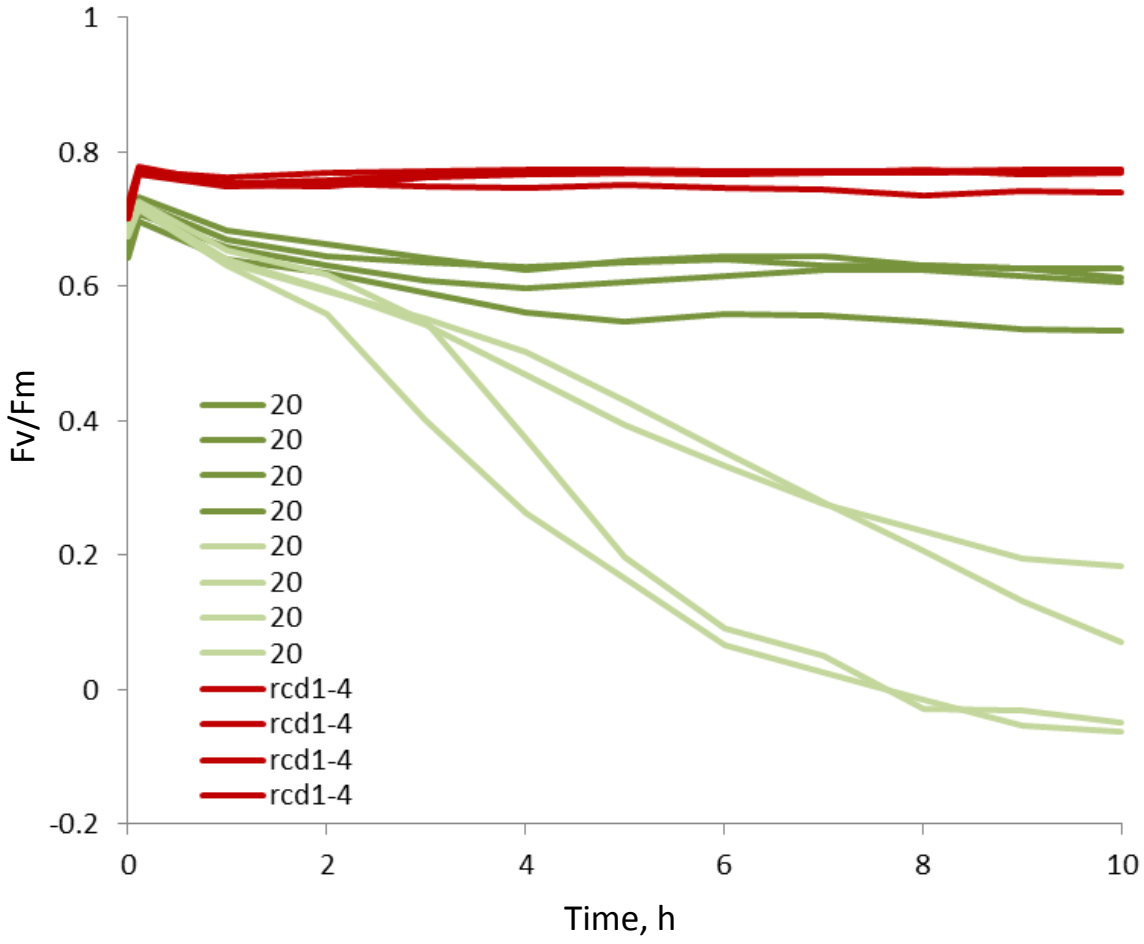

Figure 4. Unknown variation in the MV sensitivity of #20. Dark green color means an individual in the middle of the cultivation shelf. Light green means an individual in the edge of the shelf. Middle of the shelf received higher light intensity.

Figure 5A. Cultivation shelf. Middle of the shelf receives the highest light intensity.

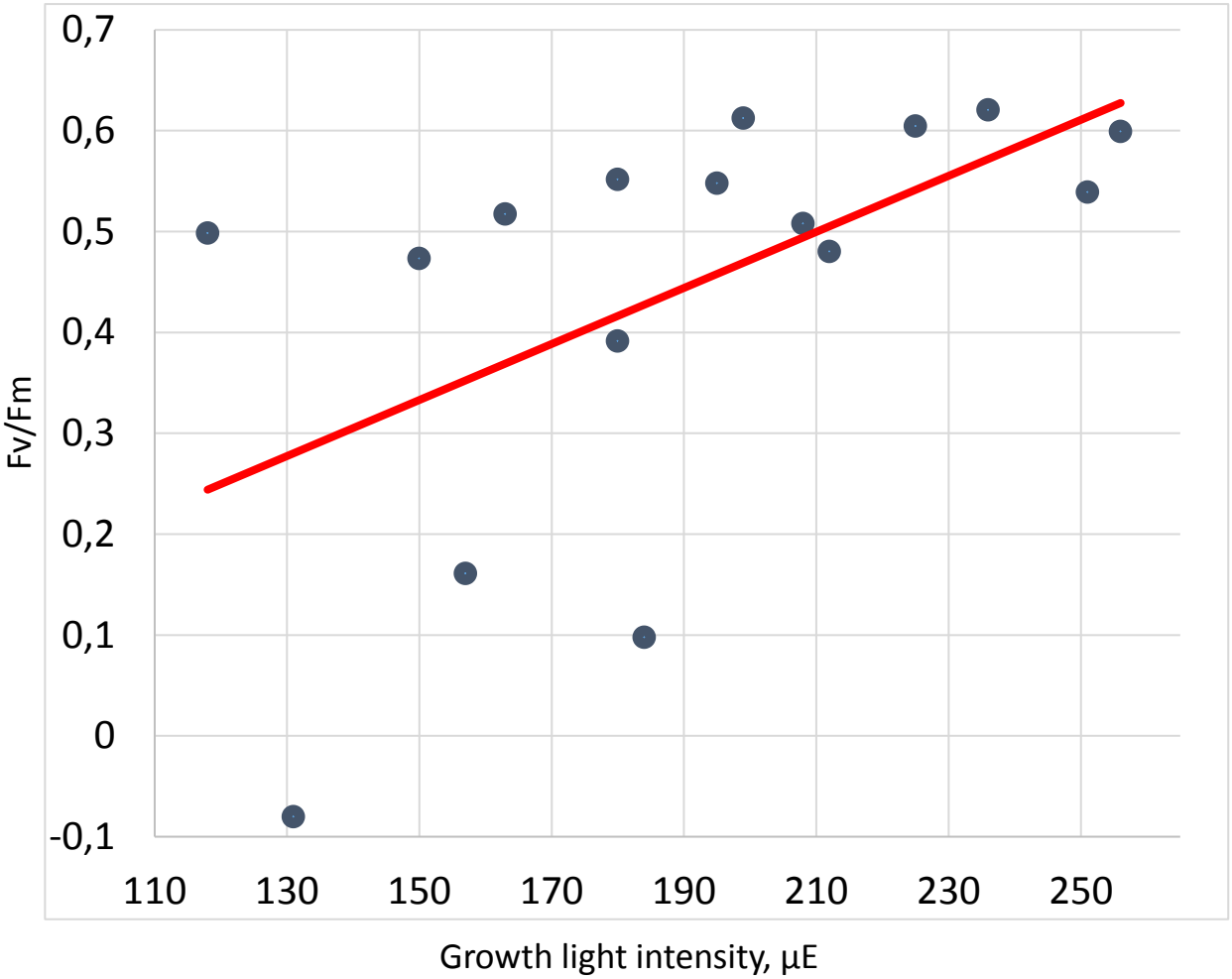

Figure 5B. The effect of growth light intensity on the MV tolerance of the screen candidate line #20. The plants grown under different light intensities as indicated on the x-axis were treated with 1 µM MV. MV tolerance was measured using PAM and plotted on y-axis. The result suggested that MV tolerance is growth light-dependent.

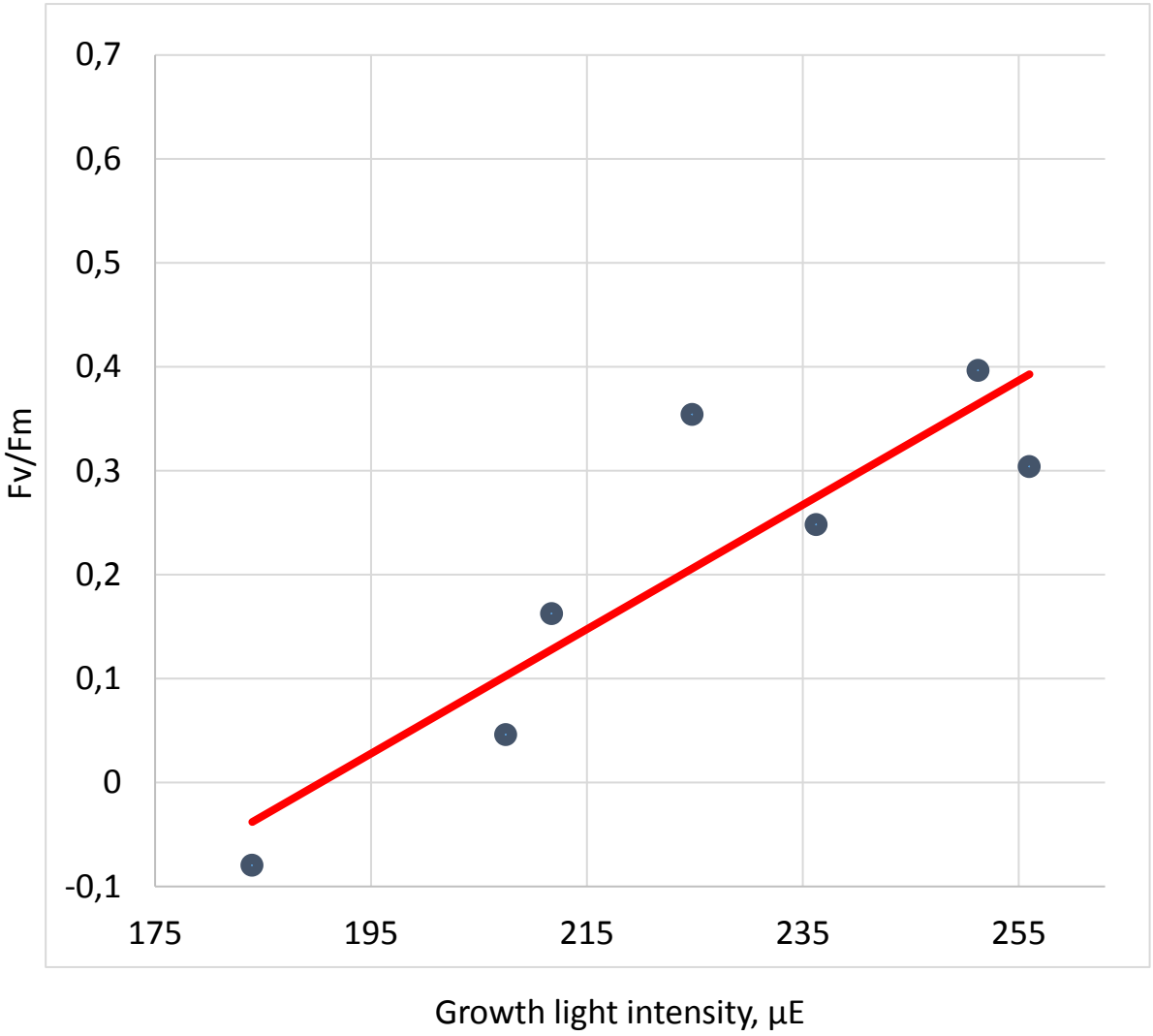

Figure 6. The effect of growth light intensity on the MV tolerance in Col-0 measured as described in Figure 5. 0,33 µM MV was used. MV tolerance is growth light-dependent.

Figure 7. #20 BcF2: small, yellowish pale and curly plants (circled) had decreased MV sensitivity. These plants were selected for sequencing directly from the phenotype.

3.6 Shoremapping and identification of *BFA1* as a possible candidate gene in line #20

Nuclear DNA sequencing of #20 revealed hundreds of EMS mutations. We expected to find an EMS frequency peak on one of the chromosomes. Indeed, it was observed on the chromosome 3 (**Figure 8**). It means the causing mutation must be there, because we selected same kind of phenotypes from the backcrossed individuals (small and pale ones that had decreased tolerance to MV).

The list of the affected genes localized in the chromosome 3 frequency peak is presented in **Figure 9**. Out of these possible causative genes, the most promising mutation had glutamine residue (Q) changing to a stop codon. It is a so-called strong mutation meaning that the gene was really disrupted. It is a probable cause for the MV tolerance. This gene is called *BFA1* (*BIOGENESIS FACTOR REQUIRED FOR ATP SYNTHASE1*) (AT3G29185). The product of this gene, the BFA1 protein, is localized in the chloroplast

stroma. It interacts with CF1β, γ, and ε subunits of the chloroplast ATP synthase, and is essential for assembly of its F_1 module (Zhang et al., 2018).

We did not see a frequency peak of mutations in any chromosome of line #54 so we couldn't find the causative gene. Thus, we focused our further research on the candidate genes from #20.

Candidate line	Gene name	Type of mutation	Aminoacid change		Candidate gene description	Predicted localization of the product	Mutant line
#20	AT3G14460	Nonsynonymous	P	S	LRR and NB-ARC domains-containing disease resistance protein;(source	nucleus	SALK_138613C
							SALK_051867C
#20	AT3G28140	Nonsynonymous	D	N	RNA ligase/cyclic nucleotide phosphodiesterase family protein;(source:	Araport11)	SALK_053448C
#20	AT3G29185	Nonsynonymous	Q	*	BFA1, BIOGENESIS FACTOR REQUIRED FOR ATP SYNTHASE 1	chloroplast	SALK_030444
							SALK_107769C (3'UTR!)
#20	AT3G43210	Nonsynonymous	R	K	ARABIDOPSIS NPK1-ACTIVATING KINESIN 2, ATNACK2, NACK2, NPK1-AC	cytoplasm, kinesin complex, microtubule,	SALK_113909C
							SALK_087097C
#54	AT3G03380	Nonsynonymous	D	N	DEG7, DEGP PROTEASE 7, DEGP7, DEGRADATION OF PERIPLASMIC PROTE	Located in chloroplast, involved in photoin	SALK_016027C
#54	AT3G03640	Nonsynonymous	S	N	BETA GLUCOSIDASE 25, BGLU25, GLUC	Located in ER	SALK_116931C
#54	AT3G13470	Nonsynonymous	V	I	CHAPERONIN-60BETA2, CPN60BETA2, CPNB2	In chloroplast	SALK_014547C
#20 and #54	AT3G22380	Nonsynonymous	Q	*	TIC, TIME FOR COFFEE	In nucleus	SAIL_753_E03
							tic-2

Figure 9. List of genes of interest in which EMS mutagenesis lead to non-synonymous aminoacid replacements in the chromosome three of line #20. BFA1 is highlighted.

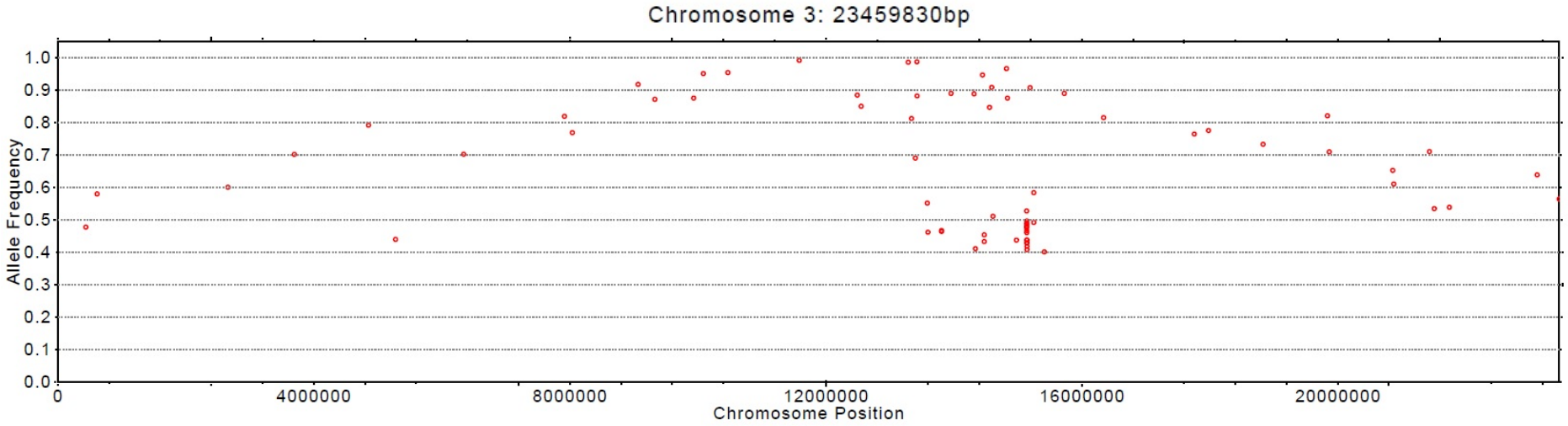

Figure 8. The frequency peak of EMS mutations in the chromosome 3 of the line #20 from the sequencing report.

3.7 Obtaining and genotyping independent mutant alleles for the assumed causative genes

From the #20 chromosome 3 frequency peak we identified possible causative genes as indicated in **Figure 9**. The independent T-DNA insertion mutants were ordered from the Arabidopsis NASC mutant collection (http://arabidopsis.info/BasicForm). The names of these mutant lines are presented in the right column of **Figure 9**. Homozygous mutations were confirmed by PCR. The dwarf and yellowish pale habitus of the homozygous mutant line SALK_030444 was similar to that of the line #20. In this mutant the gene *BFA1* (AT3G29185) was knocked out. Thus, we further refer to this mutant as *bfa1*. MV tolerance of *bfa1* was slightly lower than that of Col-0 (**Figure 10**). We crossed *bfa1* to *rcd1* and confirmed the double mutant by PCR genotyping. The *rcd1 bfa1* double mutant was significantly more sensitive to MV than *rcd1* (**Figure 10**). Both the habitus and the MV tolerance of *rcd1 bfa1* was very similar to that of #20. From these results we concluded that *BFA1* is likely the causative gene, whose disruption led to the phenotypes observed in the line #20.

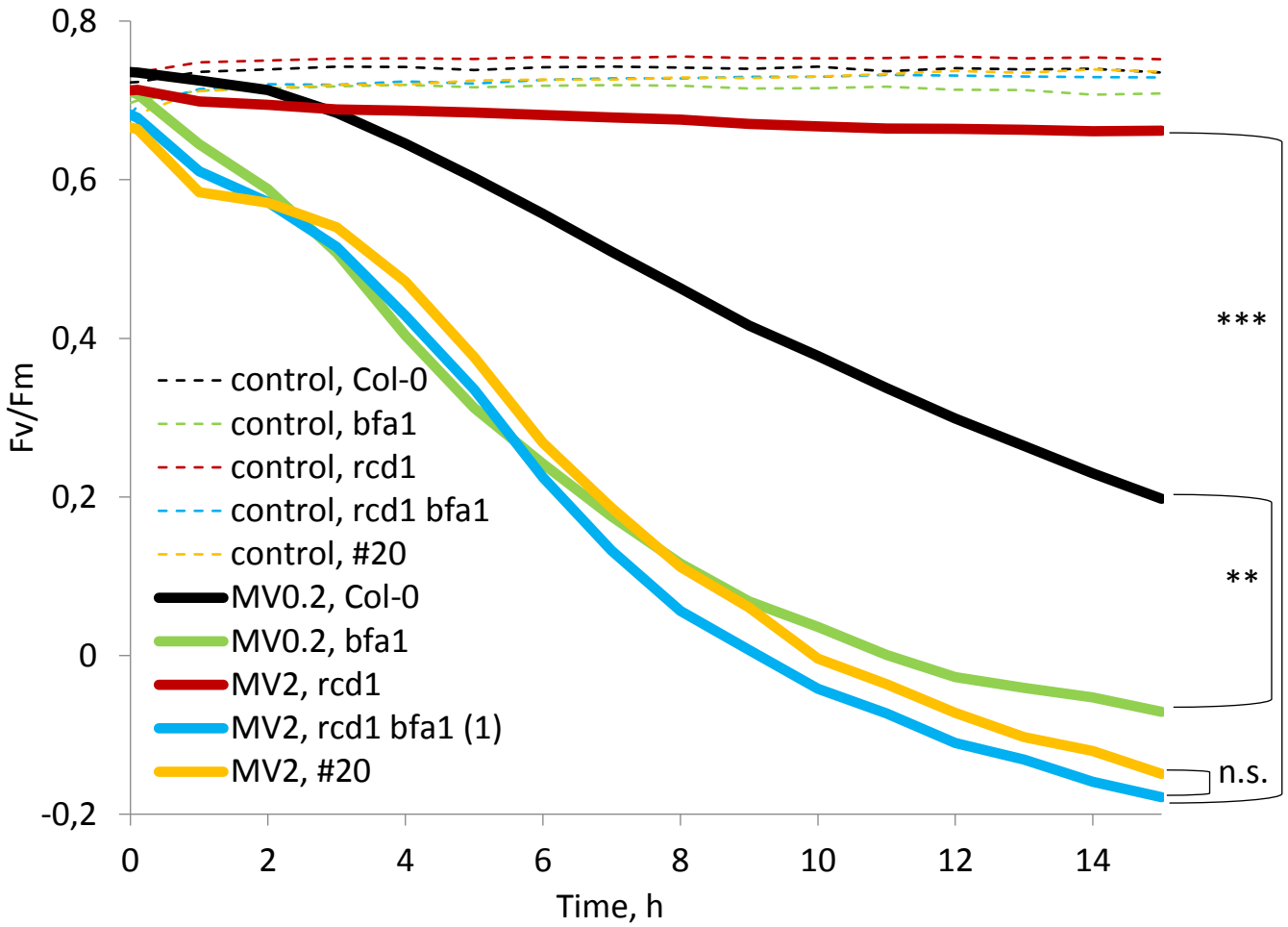

Figure 9. MV tolerance of *bfa1* and *rcd1 bfa1* estimated using PAM. Controls don't have MV in the solution, only water and Tween 20. Note that Col-0 and *bfa1* were treated with MV 0.2 µM, while *rcd1, rcd1 bfa1* and #20 were treated with MV 2 µM. Statistical analyses were performed on the last time point of the time series. *** indicates $P < 0.001$, Bonferroni post hoc correction. ** indicates $P < 0.005$, one-way ANOVA with Bonferroni pos hoc correction. Statistics presented in Appendices 1 and 2.

3.8 Abundance of BFA1 in *rcd1* determined by Western blotting

Absence of BFA1 resulted in different levels of MV sensitivity in the wild type and *rcd1* genetic backgrounds (**Figure 9**). It appeared that the absence of BFA1 led to more significant depression of MV tolerance in *rcd1* than in Col-0. The *rcd1* mutant is characterized by altered expression of many genes (Brosche et al., 2014; Shapiguzov et al., 2019). Therefore, one possible explanation for the difference could be that the levels of BFA1 expression are different in *rcd1* and in Col-0. To address this hypothesis, we decided to test abundance of BFA1 in Col-0 vs. *rcd1*. We isolated protein extracts from these lines that were grown at different light conditions. There was no striking difference in abundance of BFA1 between the genotypes under standard growth light. However, under higher light *rcd1* accumulated less and under low light possibly a bit more BFA1 than Col-0. That means that RCD1 may be involved in expression of this chloroplast component, but more research needs to be done to study this. The Western blotting also confirmed that BFA1 protein is missing in the #20 line (**Figure 11**).

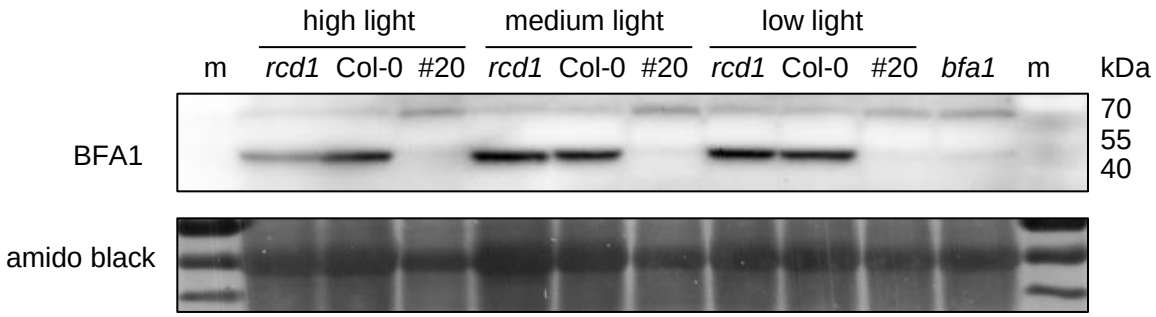

Figure 11. Abundance of BFA1 in different studied genotypes and grow conditions. It was estimated by Western blotting of total protein extracts using specific antibodies against BFA1 (Zhang et al., 2018). Amido black staining of Rubisco large subunit is presented in the bottom panel as the loading control.

4 CONCLUSIONS AND DISCUSSION

1. We did not find defects in organelle positions of *rcd1* that could explain its MV tolerance.

2. Forward genetic screen revealed that the chloroplast auxiliary protein BFA1 that is involved in assembly of ATP synthase contributes to MV tolerance of plants. Introducing *bfa1* mutation in the *rcd1* background made the plants more MV-sensitive. The future research would be to find the mechanism how BFA1 affects to chloroplastic ROS tolerance.

By using independent T-DNA insertional *bfa1* mutant we confirmed that BFA1 was likely the causative gene explaining MV sensitivity of the line #20. Similarly to the line #20, the *BFA1* homozygous knockout plants were small and pale. We tested MV tolerance of the *bfa1* line, and it was lower than in Col-0. We then crossed *bfa1* to *rcd1*. MV tolerance of *rcd1 bfa1* was lower than in *rcd1*. The *rcd*1 *bfa1* mutant was really similar to #20 in habitus and MV tolerance. We then asked our collaborators for the antibodies and did Western blot on our lines. As expected, the line #20 showed no detectable BFA1 protein, similarly to *bfa1*. The difference in BFA1 abundance between *rcd1* and Col-0 and under different growth light conditions could potentially be linked to the observed differences on MV tolerance of plants grown in different light conditions, although this requires more accurate quantification.

The activity of ATP synthase is tightly connected to the toxicity of MV, although the details are not yet clear (Shapiguzov et al., 2020). MV inhibits thylakoid proton conductivity, elevates proton motive force and decreases proton flux from stroma into the lumen. When MV-treated plants are illuminated, they trigger fast inhibition of thylakoid ATP synthase which causes rapid acidification of thylakoid lumen and increase in NPQ (Shapiguzov et al., 2020). The *bfa1* mutant also shows defects in ATP synthase activity. We therefore hypothesize that MV tolerance of *rcd1* partially depends on altered regulation of ATP synthase. But if we mutate also BFA1, this regulation of ATP synthase becomes impossible. And for this reason MV tolerance of *rcd1 bfa1* becomes

lower than in *rcd1*. Thus, my work adds important mechanistic details to the research of light stress.

ACKNOWLEDGEMENTS

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APPENDICES

Appendix 1: Figure 10 statistics: Col-0 vs. *bfa1*

```
ONEWAY VAR00002 BY VAR00001
  /MISSING ANALYSIS
  /POSTHOC=BONFERRONI ALPHA(0.05).
```

ONEWAY ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0,289	1	0,289	15,295	0,002
Within Groups	0,264	14	0,019		
Total	0,553	15			

Appendix 2: Figure 10 statistics: *rcd1* vs. *rcd1bfa1* vs. #20

```
UNIANOVA VAR00002 BY VAR00001
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
  /POSTHOC=VAR00001(BONFERRONI)
  /CRITERIA=ALPHA(0.05)
  /DESIGN=VAR00001.
```

Tests of Between-Subjects Effects

Dependent Variable: VAR00002

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.322[a]	2	1,661	532,790	0,000
Intercept	0,284	1	0,284	91,008	0,000
VAR00001	3,322	2	1,661	532,790	0,000
Error	0,062	20	0,003		
Total	3,560	23			
Corrected Total	3,384	22			

a. R Squared = .982 (Adjusted R Squared = .980)

Post Hoc Tests

VAR00001

Multiple Comparisons

Dependent Variable: VAR00002

Bonferroni

(I) VAR00001		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
rcd1	rcd1 bfa1	.8402*	0,02890	0,000	0,7647	0,9157
	#20	.8108*	0,02890	0,000	0,7353	0,8863
rcd1 bfa1	rcd1	-.8402*	0,02890	0,000	-0,9157	-0,7647
	#20	-0,0294	0,02792	0,916	-0,1023	0,0436
#20	rcd1	-.8108*	0,02890	0,000	-0,8863	-0,7353
	rcd1 bfa1	0,0294	0,02792	0,916	-0,0436	0,1023

Based on observed means.
The error term is Mean Square(Error) = .003.
*. The mean difference is significant at the 0.05 level.